

# Calcineurin in Human Heart Hypertrophy

Oliver Ritter, MD; Susanne Hack, MS; Kai Schuh, PhD; Nicola Röthlein, BA; Andreas Perrot, PhD; Karl J. Osterziel, MD; Hagen D. Schulte, MD; Ludwig Neyses, MD

**Background**—In animal models, increased signaling through the calcineurin pathway has been shown to be sufficient for the development of cardiac hypertrophy. Calcineurin activity has been reported to be elevated in the myocardium of patients with congestive heart failure. In contrast, few data are available about calcineurin activity in patients with pressure overload or cardiomyopathic hypertrophy who are not in cardiac failure.

**Methods and Results**—We investigated calcineurin activity and protein expression in 2 different forms of cardiac hypertrophy: hypertrophic obstructive cardiomyopathy (HOCM) and aortic stenosis (AS). We found that the C-terminus of calcineurin A protein containing the autoinhibitory domain was less abundant in myocardial hypertrophy than in normal heart, which suggests the possibility of proteolysis. No new splice variants could be detected by reverse-transcription polymerase chain reaction. This resulted in a significant elevation of calcineurin enzymatic activity in HOCM and AS compared with 6 normal hearts. Increased calcineurin phosphatase activity caused increased migration of NF-AT2 (nuclear factor of activated T cells 2) in SDS-PAGE compatible with pronounced NF-AT dephosphorylation in hypertrophied myocardial tissue.

**Conclusions**—Hypertrophy in HOCM and AS without heart failure is characterized by a significant increase in calcineurin activity. This might occur by (partial) proteolysis of the calcineurin A C-terminus containing the autoinhibitory domain. Increased calcineurin activity has functional relevance, as shown by altered NF-AT phosphorylation state. Although hypertrophy in AS and HOCM may be initiated by different upstream triggers (internal versus external fiber overload), in both cases, there is activation of calcineurin, which suggests an involvement of this pathway in the pathogenesis of human cardiac hypertrophy. (*Circulation*. 2002;105:2265-2269.)

**Key Words:** hypertrophy ■ cardiomyopathy ■ signal transduction

A novel signaling cascade leading to cardiac hypertrophy has recently been identified by protein interaction analysis and transgenic mouse technology.<sup>1</sup> In this model, constitutively activated calcineurin dephosphorylated the nuclear transcription factor NFAT (nuclear factor of activated T cells), enabling its nuclear translocation and resulting in induction of genes typical of cardiac hypertrophy.

See p 2242

Two studies have been performed to elucidate the relevance of this pathway in terminally failing human heart. The results were divergent, because one study revealed increased calcineurin activity,<sup>2</sup> whereas others found no alteration.<sup>3</sup> One report could demonstrate differential regulation of the calcineurin pathway and other hypertrophic signaling pathways in both compensated and decompensated hypertrophy.<sup>4</sup> In the present study, we addressed the question of whether calcineurin expression and activity are elevated in hypertrophied but nonfailing human myocardium. We investigated enzymatic activity and protein expression in cardiac tissue of

2 well-defined subsets of patients: hypertrophic obstructive cardiomyopathy with indication for myotomy/myectomy and severe aortic stenosis at the time of valve replacement.

## Methods

### Patient Population

Tissue from the left ventricular outflow tract was obtained from 13 patients undergoing aortic valve replacement for aortic stenosis and from 19 unrelated patients with hypertrophic obstructive cardiomyopathy undergoing transaortic subvalvular myotomy-myectomy<sup>5</sup> for severe obstruction of the left ventricular outflow tract. The clinical evaluation consisted of preoperative physical examination, ECG, echocardiography, and cardiac catheterization. All patients in this study showed normal ejection fractions. Tissue from the interventricular septum was taken from 6 healthy donor hearts that could not be transplanted because of technical reasons. Tissue samples were frozen in liquid nitrogen immediately after resection.

### Calcineurin Enzymatic Activity

Calcineurin enzymatic activity was measured as described elsewhere<sup>6</sup> with minor modifications. The RII-phosphopeptide (Biomol) was used as a highly specific substrate for calcineurin. The detection

Received December 11, 2001; revision received March 6, 2002; accepted March 6, 2002.

From the Department of Medicine (O.R., S.H., K.S., N.R.), University of Würzburg, Germany; Franz Volhard Clinic at the Max Delbrück Center (A.P., K.J.O.), Charité, Humboldt University of Berlin, Germany; Department of Thoracic Surgery (H.D.S.), Heinrich Heine University Düsseldorf, Germany; and University Department of Medicine (L.N.), Manchester, UK.

Correspondence to Ludwig Neyses, MD, University Department of Medicine, Manchester Heart Center, Manchester Royal Infirmary, Oxford Road, Manchester M13 9WL, UK. E-mail ludwig.neyeses@mhc.cmht.nwest.nhs.uk and labneyeses@medizin.uni-wuerzburg.de

© 2002 American Heart Association, Inc.

*Circulation* is available at <http://www.circulationaha.org>

DOI: 10.1161/01.CIR.000016044.19527.96

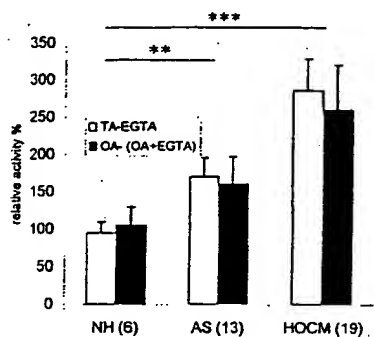


Figure 1. Quantification of calcineurin phosphatase activity. Two approaches were applied. (1) Calcineurin activity was calculated as total phosphatase activity (TA) minus activity in presence of EGTA. (2) Calcineurin activity was determined as phosphate released in presence of okadaic acid (OA) minus phosphate released in presence of OA plus EGTA. There was no significant difference between the 2 approaches (correlation coefficient  $r=0.93$ ). NH indicates normal heart ( $n=6$ ); AS, aortic stenosis ( $n=13$ ); and HOCM, hypertrophic obstructive cardiomyopathy ( $n=19$ ). \* $P<0.01$ ; \*\* $P<0.001$ .

of free phosphate released from RII by calcineurin was based on the malachite green dye reaction. Calcineurin enzymatic activity was measured by 2 independent methods, as described below.

Calcineurin requires  $\text{Ca}^{2+}$  for its activity; thus, in a first series of experiments, EGTA (20 mmol/L) was added to the assay. Calcineurin activity was calculated as total (RII-) phosphatase activity minus activity in the presence of EGTA. However, the phospho group may be cleaved by other competing phosphatases. Thus, a second series of experiments was performed. Okadaic acid inhibits protein phosphatases 1 and 2A (PP1, PP2A) but not calcineurin. Therefore, calcineurin activity was determined as phosphate released from RII in the presence of okadaic acid minus phosphate released in the presence of okadaic acid plus EGTA.

Reactions were terminated after 30 minutes, and absorption was read on a microtiter plate reader at 620 nm. Comparison of the 2 methods yielded comparable results (Figure 1).

### Calcineurin Protein Expression

Proteins were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with monoclonal antibodies directed against the N-terminus (catalytic domain) and the C-terminus (autoinhibitory domain). The antibody against the N-terminal half (StressGen) recognized both calcineurin A (CnA)- $\alpha$  and CnA- $\beta$ . The antibodies against the C-terminus (St. Cruz) were directed specifically against either the  $\alpha$ - or  $\beta$ -isoform of the calcineurin subunit A. Further processing was according to standard procedures.<sup>7</sup> Protein was visualized with the ECL detection system (Amersham Pharmacia Biotech). Signals were quantified with ScanPack (Biometra).

### Calcineurin Reverse-Transcription Polymerase Chain Reaction Analysis

Total mRNA from 6 patients from each group was isolated by single-step RNA isolation with the Trizol reagent (Life Technologies). Analysis of calcineurin mRNA levels from normal and hypertrophied human myocardium was performed by semiquantitative 1-step reverse-transcription polymerase chain reaction (PCR; Qiagen) according to the manufacturer's protocol. Oligonucleotide sequences for calcineurin were CTATCCAGCAGTGTGTGA and CTGAGGCACAGCAAGTTGC according to published sequences (GeneBank accession No. XM 011860).

### NF-AT Phosphorylation

Calcineurin activity was assessed by evaluation of NF-AT phosphorylation. Proteins were separated by 6% SDS-PAGE and transferred

to nitrocellulose membranes. The membranes were incubated with monoclonal antibodies (St. Cruz) directed against NF-AT2. As positive control, crude tissue extracts of normal heart were incubated with human recombinant calcineurin (Biomol).

### Statistical Analysis

All data are presented as mean  $\pm$  SEM. Differences among groups were compared by ANOVA. Significance was assigned a value of  $P<0.05$ . Correlation coefficient  $r$  was calculated by Pearson's test.

## Results

### Calcineurin Activity

We investigated calcineurin phosphatase activity in 13 patients with aortic stenosis and in 19 patients with hypertrophic obstructive cardiomyopathy. In patients with aortic stenosis, calcineurin activity was increased to  $163 \pm 11\%$  ( $P<0.01$ ), ranging from 111% to 217% (calcineurin phosphatase activity in 6 normal hearts was set as 100%). In cardiomyopathic patients, calcineurin activity was elevated to  $275 \pm 13\%$  ( $P<0.001$ ), ranging from 112% to 336% (Figure 1).

### Calcineurin Protein Expression

Western blot analysis was performed to investigate calcineurin protein expression (Figure 2). When an antibody specific for the N-terminal part (catalytic domain) was used, signals of CnA protein expression were increased in hypertrophied myocardium compared with normal heart. In aortic stenosis, CnA protein level was increased to  $151 \pm 27\%$  (range 111% to 223%;  $P<0.05$ ). In hypertrophic obstructive cardiomyopathy, CnA protein expression was increased to  $178 \pm 38\%$  (range 113% to 278%;  $P<0.01$ ; Figure 3A).

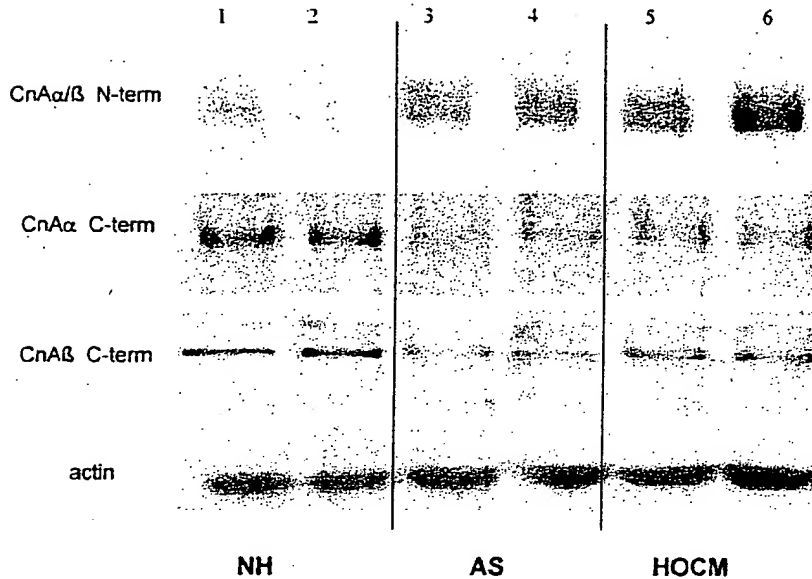
When an antibody specific for the C-terminus (autoinhibitory domain) was used, signals were decreased compared with normal heart. In aortic stenosis, CnA- $\alpha$  protein level was decreased to  $60 \pm 8\%$  (range 47% to 69%;  $P<0.01$ ). CnA- $\beta$  protein was decreased to  $68 \pm 10\%$  (range 43% to 86%;  $P<0.01$ ) compared with normal heart (100%). In hypertrophic obstructive cardiomyopathy, CnA- $\alpha$  was decreased to  $53 \pm 7\%$  (range 17% to 63%;  $P<0.01$ ) and CnA- $\beta$  to  $50 \pm 11\%$  (range 22% to 59%;  $P<0.01$ ) of the concentration in normal heart (Figure 3B).

### Calcineurin mRNA Levels

Reverse-transcription PCR from CnA was performed to investigate whether splice variants exist in addition to full-length cDNA in normal and hypertrophied myocardium. Oligonucleotides were selected to include the junction region of the N- and the C-terminus. We found complete calcineurin mRNA that included the 3'-region coding for the C-terminus in all patient groups, which indicates that degradation of the CnA C-terminus is a posttranslational process (Figure 4).

### NF-AT Phosphorylation

Activated calcineurin directly binds to NF-AT transcription factors, resulting in NF-AT dephosphorylation and translocation to the nucleus. Therefore, altered NF-AT phosphorylation states reflect increased calcineurin enzymatic activity. Migration velocity of NF-AT2 from hypertrophied myocardial tissue (hypertrophic obstructive cardiomyopathy and



**Figure 2.** Representative expression of calcineurin in different subsets of cardiac hypertrophy. Lanes 1 and 2, normal heart (NH); lanes 3 and 4, aortic stenosis (AS); lanes 5 and 6, hypertrophic obstructive cardiomyopathy (HOCM). For immunodetection, 20  $\mu$ g of total protein was used in each lane. A, Calcineurin was probed with antibody specific for N-terminal half of CnA. Antibody was not isoform specific. However, in AS and HOCM, 2 bands were detectable with this antibody, which indicates potential partial proteolysis of C-terminus. Antibodies recognizing C-terminus of (B) CnA- $\alpha$  or (C) CnA- $\beta$  were used to investigate potential proteolysis of C-terminus. Loading control (D) was performed with actin.

aortic stenosis) was increased compared with normal heart and identical to the NF-AT2 migration velocity of normal heart extracts treated with additional external calcineurin. This suggests an altered NF-AT phosphorylation state in hypertrophied myocardium (Figure 5).

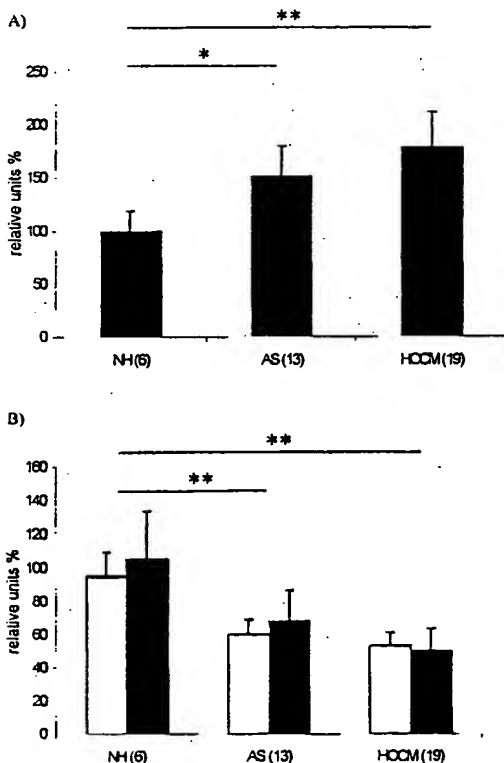
## Discussion

In the compensated phase of human myocardial hypertrophy, we found a reduction of the CnA C-terminus (containing the autoinhibitory domain) compatible with partial proteolysis and increased calcineurin enzymatic activity compared with normal hearts. This amounted to a 2.8-fold increase in calcineurin activity in the myocardium of patients with hypertrophic obstructive cardiomyopathy. In aortic stenosis, there was a 1.6-fold increase in calcineurin activity.

A major obstacle in the elucidation of the pathogenesis of human cardiac hypertrophy is the heterogeneity of its causes: physiological (eg, in athletes) versus pathological hypertrophy with its many variations, eg, in hypertension, aortic stenosis, and hypertrophic cardiomyopathy. To reduce some of this variability, we chose to focus on 2 well-defined and relatively homogeneous forms of hypertrophy: hypertrophic obstructive cardiomyopathy and aortic stenosis before the onset of heart failure.

A number of recent studies investigated calcineurin activity in different animal models of cardiac hypertrophy.<sup>1,6,8-11</sup> It was shown that calcineurin activity was increased in several animal lines with transgenic overexpression of mutated sarcomeric proteins and in some animal models with external overload. However, 2 groups<sup>12,13</sup> found no changes in calcineurin activity, and another group<sup>14</sup> reported decreased calcineurin activity. The disparate results might have been caused by differences in the experimental protocols (eg, in the antibodies used; see our results) or might have reflected truly distinct roles of calcineurin in the pathogenesis of these disease models.

In the human heart, calcineurin phosphatase activity under conditions of increased workload has been discussed controversially. Two studies investigated activation of the calcineurin pathway in terminally failing hearts. One group observed an increase in calcineurin activity<sup>2</sup> using a calcineurin immunoprecipitation assay. Another group reported a decrease in calcineurin protein expression<sup>3</sup> with an antibody specific for the CnA C-terminus.



**Figure 3.** A, CnA protein expression in human hypertrophic myocardium. When antibody for N-terminus, immunoreactive with CnA- $\alpha$  and - $\beta$ , was used, there was clear increase in calcineurin expression in hypertrophied myocardium. \* $P < 0.05$ ; \*\* $P < 0.01$ . B, CnA protein expression in human hypertrophic myocardium. Antibodies for C-terminus of CnA ( $\alpha$  and  $\beta$ ) revealed decrease compared with normal heart. \*\* $P < 0.01$ .

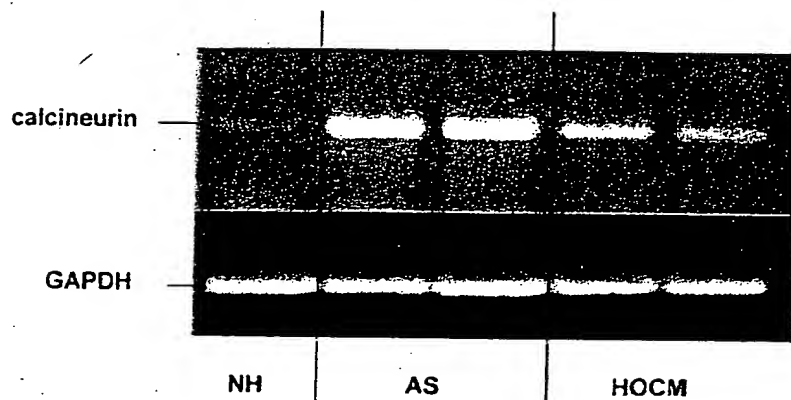


Figure 4. Representative example of CnA- $\beta$  transcripts. Reverse-transcription PCR was performed to investigate calcineurin mRNA. Resulting PCR products were separated in 1% agarose gel and stained with ethidium bromide. In each patient group, ie, hypertrophic cardiomyopathy (HOCM), aortic stenosis (AS), and normal heart (NH), intact calcineurin mRNA was demonstrated that spanned the catalytic domain and the autoinhibitory domain. No splice variants were detectable. Control PCR was performed with GAPDH.

In addition to the calcineurin pathway, there are other signaling cascades involved in the development and maintenance of myocardial hypertrophy and in the transition from hypertrophy to failure. The mitogen-activated protein kinases and the protein kinase Akt were found to be elevated in heart failure but not in cardiac hypertrophy. In contrast, calcineurin was activated both in hypertrophy and failure.<sup>4</sup> This was accompanied by an increase in full-length calcineurin protein expression. No degradation of the C-terminus was described. However, the above-mentioned studies were performed on patients with hypertensive, ischemic, or dilated cardiomyopathy.

In contrast, in patients with myocardial hypertrophy but with compensated ventricular function caused by aortic stenosis or hypertrophic obstructive cardiomyopathy, we here report results compatible with the hypothesis of cleavage of the C-terminus of calcineurin A, which contains an autoinhibitory domain. This is accompanied by an increase in calcineurin phosphatase activity. Our results are in line with 2 recent studies on rat myocardium that describe increased calcineurin activity in physiological hypertrophy<sup>10</sup> and at the beginning of pressure-overload hypertrophy,<sup>6</sup> although neither group was investigating proteolysis of calcineurin in these animals.

Calcineurin is a heterodimer of a 59-kDa subunit, CnA, and a 19-kDa subunit, CnB. This subunit is tightly bound to CnA. CnA consists of a catalytic and a regulatory domain. The regulatory domain contains the CnB binding domain, the

calmodulin binding domain, and an autoinhibitory domain at the C-terminus (reviewed in Klee et al<sup>15</sup>).  $\text{Ca}^{2+}$  binding to CnB enables calmodulin binding to CnA, which results in displacement of the autoinhibitory domain from the catalytic subunit, thus activating the enzyme.<sup>15</sup> The catalytic domain is resistant to proteolysis,<sup>16</sup> whereas the regulatory domain at the C-terminus containing the autoinhibitory domain is sensitive to proteolysis.<sup>16,17</sup>

In the study presented here, an antibody specific for the N-terminal half of CnA detected 2 bands with different molecular weight in hypertrophied myocardium but not in normal heart, which indicates partial degradation of CnA, possibly on the C-terminus. This is substantiated by the fact that in normal heart, there is a stronger signal for the CnA C-terminus in Western blots than in hypertrophied myocardium. Recent evidence suggests that in ischemia-reperfusion injury, calcium overload may activate calpains, resulting in selective proteolysis of myofibrils.<sup>18</sup> Increased intracellular calcium in myocardial hypertrophy may act in a related manner and therefore may lead to proteolysis of cytosolic proteins. Indeed, proteolysis of calcineurin by calpain I increased phosphatase activity dramatically.<sup>19</sup>

A problem pertinent to all studies in human cardiac tissue is whether maximal calcineurin activity measured in tissue homogenates faithfully reflects *in vivo* activity. At present, this cannot be answered directly, but our indirect measurements of calcineurin activation such as NF-AT phosphorylation status may be a first clue to altered NF-AT phosphorylation status *in vivo*. NF-AT enters the nucleus when calcineurin dephosphorylates critical serines in the N-terminus of NF-AT.<sup>20</sup> It has been shown that NF-AT is dephosphorylated on 13 residues on stimulation.<sup>21</sup> We found increased electrophoretic mobility of NF-AT2 in hypertrophied myocardial tissue, which indicates pronounced dephosphorylation of NF-AT2 in hypertrophied myocardium compared with normal heart. These findings are compatible with the concept that increased calcineurin activity is indeed relevant in myocardial hypertrophy.

Moreover, calcineurin binding proteins have recently been discovered that inhibit calcineurin phosphatase activity (overview in Leinwand<sup>22</sup>). These proteins, especially myocyte-enriched calcineurin-interacting protein-1,<sup>23</sup> may alter calcineurin signaling in the myocardium of different disease

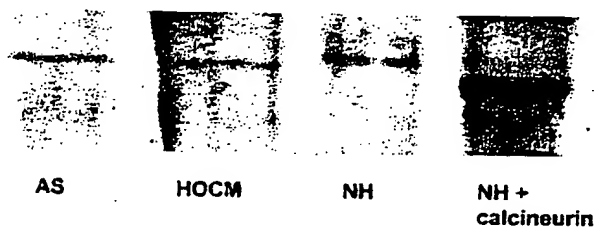


Figure 5. Representative Western blot demonstrating different NF-AT2 phosphorylation states in different myocardial tissues. Lane 1, aortic stenosis (AS); lane 2, hypertrophic cardiomyopathy (HOCM); lane 3, normal heart (NH); lane 4, NH with human recombinant calcineurin. NF-AT2 from hypertrophied myocardial tissue is supposed to be dephosphorylated, resulting in increased migration velocity on 6% SDS gel. Addition of abundant calcineurin to normal heart extracts also dephosphorylates NF-AT2.

groups. It will be interesting to measure expression of these proteins once antibodies have become generally available.

To the best of our knowledge, this is the first study investigating calcineurin in the following subsets of patients with cardiac hypertrophy: hypertrophic obstructive cardiomyopathy and severe aortic stenosis. In view of the relative rarity of these patients (particularly hypertrophic obstructive cardiomyopathy with indication for operation), the numbers are large. Of note, large pieces of tissue were used for measurements as opposed to percutaneous biopsy samples with the inherent problem of tissue heterogeneity (fibrosis). These 2 factors may also explain the relatively low variability of our results compared with other studies in human tissue.

In summary, our results are compatible with an activation of the calcineurin pathway in these 2 subsets of human cardiac hypertrophy and strengthen the concept that calcineurin is involved in the pathogenesis of human cardiac hypertrophy.

### References

1. Molkentin JD, Lu JR, Antos CL, et al. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell*. 1998;93:215-228.
2. Lim HW, Molkentin JD. Calcineurin and human heart failure. *Nat Med*. 1999;5:246-247.
3. Tsao L, Neville C, Musaro A, et al. Revisiting calcineurin and human heart failure. *Nat Med*. 2000;6:2-3.
4. Haq S, Choukroun G, Lim H, et al. Differential activation of signal transduction pathways in human hearts with hypertrophy versus advanced heart failure. *Circulation*. 2001;103:670-677.
5. Schulte HD, Bircks WH, Loesse B, et al. Prognosis of patients with hypertrophic obstructive cardiomyopathy after transaortic myectomy: late results up to twenty-five years. *J Thorac Cardiovasc Surg*. 1993;106:709-717.
6. Lim HW, De Windt LJ, Steinberg L, et al. Calcineurin expression, activation, and function in cardiac pressure-overload hypertrophy. *Circulation*. 2000;101:2431-2437.
7. Hammes A, Oberdorf-Maass S, Rother T, et al. Overexpression of the sarcolemmal calcium pump in the myocardium of transgenic rats. *Circ Res*. 1998;83:877-888.
8. Sussman MA, Lim HW, Gude N, et al. Prevention of cardiac hypertrophy in mice by calcineurin inhibition. *Science*. 1998;281:1690-1693.
9. Shimoyama M, Hayashi D, Takimoto E, et al. Calcineurin plays a critical role in pressure overload-induced cardiac hypertrophy. *Circulation*. 1999;100:2449-2454.
10. Eto Y, Yonekura K, Sonoda M, et al. Calcineurin is activated in rat hearts with physiological left ventricular hypertrophy induced by voluntary exercise training. *Circulation*. 2000;101:2134-2137.
11. Hayashida W, Kihara Y, Yasaka A, et al. Cardiac calcineurin during transition from hypertrophy to heart failure in rats. *Biochem Biophys Res Commun*. 2000;273:347-351.
12. Meguro T, Hong C, Asai K, et al. Cyclosporine attenuates pressure-overload hypertrophy in mice while enhancing susceptibility to decompensation and heart failure. *Circ Res*. 1999;84:735-740.
13. Zhang W, Kowal RC, Rusnak F, et al. Failure of calcineurin inhibitors to prevent pressure-overload left ventricular hypertrophy in rats. *Circ Res*. 1999;84:722-728.
14. Ding B, Price RL, Borg TK, et al. Pressure overload induces severe hypertrophy in mice treated with cyclosporine, an inhibitor of calcineurin. *Circ Res*. 1999;84:729-734.
15. Klee CB, Ren H, Wang X. Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. *J Biol Chem*. 1998;273:13367-13370.
16. Hubbard MJ, Klee CB. Functional domain structure of calcineurin A: mapping by limited proteolysis. *Biochemistry*. 1989;28:1868-1874.
17. Yang SA, Klee CB. Low affinity Ca<sup>2+</sup>-binding sites of calcineurin B mediate conformational changes in calcineurin A. *Biochemistry*. 2000;39:16147-16154.
18. Bolli R, Marban E. Molecular and cellular mechanisms of myocardial stunning. *Physiol Rev*. 1999;79:609-634.
19. Wang KK, Roufogalis BD, Villalobo A. Characterization of the fragmented forms of calcineurin produced by calpain I. *Biochem Cell Biol*. 1989;67:703-711.
20. Rao A, Luo C, Hogan PG. Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol*. 1997;15:707-747.
21. Okamura H, Aramburu J, Garcia-Rodriguez C, et al. Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. *Mol Cell*. 2000;6:539-550.
22. Leinwand LA. Calcineurin inhibition and cardiac hypertrophy: a matter of balance. *Proc Natl Acad Sci U S A*. 2001;98:2947-2949.
23. Rothermel BA, McKinsey TA, Vega RB, et al. Myocyte-enriched calcineurin-interacting protein, MCIPI, inhibits cardiac hypertrophy in vivo. *Proc Natl Acad Sci U S A*. 2001;98:3328-3333.